Journal of Applied Microbiology 2004, **96,** 521–534 doi:10.1111/j.1365-2672.2004.02171.x

Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their *in vitro* and *in situ* activity

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2003/0809: received 11 September 2003, revised and accepted 30 October 2003

ABSTRACT

A. CORSETTI, L. SETTANNI AND D. VAN SINDEREN. 2004.

Aims: To identify and characterize bacteriocion-producing lactic acid bacteria (LAB) in sourdoughs and to compare *in vitro* and *in situ* bacteriocin activity of sourdough- and nonsourdough LAB.

Methods and Results: Production of antimicrobial compounds by 437 *Lactobacillus* strains isolated from 70 sourdoughs was investigated. Five strains (*Lactobacillus pentosus* 2MF8 and 8CF, *Lb. plantarum* 4DE and 3DM and *Lactobacillus* spp. CS1) were found to produce distinct bacteriocin-like inhibitory substances (BLIS). BLIS-producing *Lactococcus lactis* isolated from raw barley showed a wider inhibitory spectrum than sourdough LAB, but they did not inhibit all strains of the key sourdough bacterium *Lb. sanfranciscensis*. Antimicrobial production by *Lb. pentosus* 2MF8 and *Lc. lactis* M30 was also demonstrated *in situ*.

Conclusions: BLIS production by sourdough LAB appears to occur at a low frequency, showing limited inhibitory spectrum when compared with BLIS-producing *Lc. lactis*. Nevertheless, they are active BLIS producers under sourdough and bread-making conditions.

Significance and Impact of the Study: The activity of BLIS has been demonstrated *in situ*. It may influence the complex sourdough microflora and support the implantation and stability of selected insensitive bacteria, such as *Lb. sanfranciscensis*, useful to confer good characteristics to the dough.

Keywords: bacteriocins, BLIS, *Lactobacillus pentosus*, *Lactobacillus sanfranciscensis*, *Lactococcus lactis*, sourdough.

INTRODUCTION

Lactic acid bacteria (LAB) occur naturally in several raw materials (e.g. milk, meat, flour, etc.) used to produce foods (Rodríguez *et al.* 2000). LAB are used as 'natural' or 'selected' starters in food fermentations in which they perform both acidification, due to the production of lactic and acetic acids, flavour-compound production, as well as protection of the food from spoilage and pathogenic micro-organisms by producing organic acids, hydrogen peroxide, diacetyl (Atrih *et al.* 2001; Messens and De Vuyst 2002), antifungal compounds such as fatty acids (Corsetti *et al.* 1998) or phenyllactic acid (Lavermicocca *et al.* 2000), and/or bacteriocins (De Vuyst and Vandamme 1994a).

Bacteriocins are antimicrobial peptides or small proteins which inhibit, by a bactericidal or bacteriostatic mode of action, micro-organisms that are usually closely related to the producer strain (De Vuyst and Vandamme 1994b; Schillinger and Holzapfel 1996). A bacteriocin producer protects itself against its own antimicrobial compound by means of a system referred as immunity, which is expressed concomitantly with the antimicrobial peptide (Nes *et al.* 1996). Bacteriocins from LAB have been classified, initially by Klaenhammer (1993), in four classes on the basis of common, mainly structural, characteristics. In a later review, Nes *et al.* (1996) restricted the LAB-produced bacteriocins to three classes because the existence of a fourth class as

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proposed by Klaenhammer (1993), i.e. complex compounds needing a carbohydrate or lipid moiety for activity, had been based on unpurified, and therefore ill-defined, compounds. Class I are small, heat-stable peptides containing thioether amino acids, like lanthionine, and are for this reason named lantibiotics. Class II are small, hydrophobic, heat-stable, nonmodified bacteriocins consisting of either a single peptide with antilisterial activity (class IIa) or two polypeptide chains (class IIb), and also include other peptide bacteriocins (class IIc). Class III consist of large, hydrophilic, heat-labile proteins (Klaenhammer 1993; Vaughan *et al.* 2001; Eijsink *et al.* 2002; Messens and De Vuyst 2002). Even if chemical, enzymatic or physical characteristics of the food, food processing, or the physiological state of the bacteriocin-producing micro-organism can limit the bacteriocin activity *in situ* (Eckner 1992; Messens and De Vuyst 2002), it has been claimed that micro-organisms producing bacteriocins possess a competitive advantage over other organisms living in the same natural environment (Vaughan *et al.* 2001).

Sourdoughs are considered extremely complex ecosystems where LAB and yeasts represent the prevailing microflora (Corsetti *et al.* 1996). In this system the synthesis of bacteriocins and other antimicrobial molecules could regulate the complex interactions within the starter micro-organisms and between the starter and the contaminant microflora of the sourdough (Corsetti *et al.* 1996; Hammes and Gänzle 1997). It has been reported that if sourdoughs undergo considerable propagation, this will lead to the emergence of one or two species, which prevail at three or four orders of magnitude above the contaminating microflora (Hammes *et al.* 1996; Hammes and Gänzle 1997).

In this context, besides particular adaptative responses to the conditions prevailing during sourdough fermentation (e.g. preferred utilization of maltose, the more available fermentable flour carbohydrate, high efficiency in the co-metabolism of maltose or glucose with various substrates) (Gobbetti and Corsetti 1997), the production of bacteriocins by sourdough LAB could provide another selective advantage for the producer strains (Hammes and Gänzle 1997). Some nonorganic acid inhibitory substances have been so far discovered from sourdough LAB and include the bacteriocins bavaricin A (Larsen *et al.* 1993), plantaricin ST31 (Todorov *et al.* 1999), the bacteriocin-like inhibitory substance (BLIS) C57 (Corsetti *et al.* 1996) and the small molecular weight antibiotic reutericyclin (Gänzle *et al.* 2000). Nevertheless, the role of bacteriocins under conditions that truly reflect sourdough fermentation remains to be determined, while the effective use of bacteriocins or bacteriocin-producing LAB in sourdough production and leavened baked product preservation relies on the isolation and characterization of bacteriocins from sourdough-adapted LAB, and on the understanding of the activity of these antimicrobial substances in the natural chemical–physical conditions of sourdough fermentation (Messens and De Vuyst 2002). Moreover, new bacteriocins could be discovered with activity against cereal-associated and spoilage or, eventually, pathogenic micro-organisms. In the latter case, the possible use of bacteriocins in association with other preservatives for controlling food spoilage micro-organisms could reduce the concentration of 'non-natural' preservatives, thus meeting the consumer's wishes for minimally processed foods (Rasch and Knochel 1998).

The aim of this study was to: (i) evaluate the presence of bacteriocion-producing LAB isolated from a large number of sourdoughs used in bread-making at artisanal and industrial level, (ii) characterize the bacteriocins for their inhibitory spectrum and for their *in vitro* and *in situ* (during sourdough fermentation) activity, (iii) compare the *in vitro* and *in situ* bacteriocin activity of sourdough- and nonsourdough-isolated LAB.

MATERIALS AND METHODS

Sourdough samples and isolation and identification of LAB

Eighteen wheat sourdoughs were collected from artisanal and industrial bakeries of central Italy (Marche, Toscana and Lazio regions). Three rye sourdoughs were obtained from a semi-industrial bakery from Germany. Isolation and identification of LAB from sourdoughs were performed as previously described (Corsetti *et al.* 2001).

Strains and growth conditions

Lactobacillus sanfranciscensis (85 strains), *Lb. plantarum* (34 strains), *Lb. alimentarius* (33 strains), *Lb. brevis* (30 strains), *Lb. farciminis* (13 strains), *Lb. fructivorans* (nine strains), *Lb. fermentum* (five strains), *Lb. hilgardii* (three strains), *Lb. pentosus* (two strains), *Lb. pontis* (two strains), and 221 unidentified *Lactobacillus* strains isolated from 70 wheat and rye sourdoughs (Gobbetti *et al.* 1994; Corsetti *et al.* 2001; this paper), forming a total of 437 strains, were screened for bacteriocin production. *Lactobacillus sanfranciscensis* strains were cultured in sourdough bacteria (SDB) broth (Kline and Sugihara 1971) at 30°C for approx. 18 h prior to use. All other lactobacilli were cultivated in modified-MRS (mMRS) (maltose and fresh yeast extract were added at final concentrations of 1 and 10%, respectively, and the final pH was adjusted to 5·6) at 30°C for approx. 18 h. Two bacteriocin-producing strains of *Lactococcus lactis* belonging to the National Food Biotechnology Centre (NFBC) culture collection (University College Cork, Ireland), were included in this study in order to compare their inhibitory activity with that of sourdough LAB. A nonbacteriocin-producing strain of *Lc. lactis* (strain Q13) isolated from sourdough was used as

negative control. Lactococci were cultured in M17 (Difco Laboratories, Detroit, MI, USA) broth at 30°C for 24 h. The indicator strains, listed in Table 1, were propagated as follows: lactobacilli in SDB or in MRS (De Man *et al.* 1960) at 30°C for 24 h except for *Lb. sakei*, for which a mMRS was used, and *Lb. delbrueckii* ssp. *bulgaricus*, incubated at 37°C; *Streptococcus thermophilus* in M17 (Difco) at 37°C for 24 h; *Listeria innocua* in TSBYE (Tryptone Soy Broth supplemented with 0·6% (w/v) yeast extract) (Difco) at 30°C for 24 h; *Bacillus* strains in BN (Difco) at 30°C for 24 h; and yeasts in Wallerstein Lab (WL) (Difco) at 30°C for 24 h. The indicator strains *Lb. sakei* LMG 2313 (obtained from the Laboratory of Microbial Gene Technology, Ås, Norway) and *L. innocua* 4202 belonged to the culture collection of the NFBC; all other strains were obtained from the culture collection of the Department of Food Science, Section of Food Technology and Biotechnology, Laboratory of Microbiology (University of Perugia, Italy). With the exception of DSM and ATCC strains, and of *Lb. sakei*, *Lb. casei* ssp. *pseudoplantarum*, *Lb. casei* subsp. *casei*, *Lb. curvatus*, *L. innocua*, *S. thermophilus*, and *Bacillus* spp. all other strain used as indicators, listed in Table 1, were previously isolated from sourdoughs (Gobbetti *et al.* 1994; Corsetti *et al.* 1998, 2001).

Assays for bacteriocin activity

The antimicrobial activity of lactobacilli was detected by two methods, the agar-spot deferred test and the well diffusion assay (Schillinger and Lücke 1989). In the first case, colonies of the strains to be tested for bacteriocin production were grown on the surface of SDB or mMRS containing 1·5% agar for 24 h at 30°C. The indicator strains *Lb. farciminis* CC10, *Lb. sakei* LMG 2313, *L. innocua* 4202 and *Lb. delbrueckii* subsp. *bulgaricus* B397, were inoculated (4% v/v) into 7 ml of soft agar medium (containing 0·7% agar) specific for each strain, at a final cellular concentration of $1{\cdot}5 \times 10^5$ CFU ml^{-1}. The soft media were poured on the plate where growth of the producers occurred and the plates were incubated anaerobically, at the optimal growth temperature for the indicator strains, for 24 h. Inhibition was scored positive in presence of a detectable clearing zone around the colony of the producer strain.

For the well diffusion assay, agar base medium (1·5% agar) was overlaid with soft agar medium containing the indicator strain, as reported above. Thereafter wells (10 mm in diameter) were cut into the agar and 200 l of the cell-free supernatant of the potential producer strains was placed into each well. In order to eliminate the inhibitory effect of lactic acid and/or H_2O_2, the supernatants were adjusted to pH 6·5 with 1 mol l^{-1} NaOH and treated with catalase (1 mg ml^{-1}), followed by filtration through a 0·22-μm pore size filter (Nucleopore, Costar Corporation, Cambridge, MA, USA). Prior to incubation for 24 h at the optimal growth temperature for the indicator strains, plates were refrigerated (4°C) for 4 h to allow the radial diffusion of the compounds contained in the supernatant.

The antibacterial activity of the supernatants was evaluated by the critical dilution assay of Barefoot and Klaenhammer (1983). Bacteriocin activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strains and was expressed as activity units per millilitre (AU ml^{-1}).

Phenotypic and genotypic identification of lactobacilli

Unidentified Gram-positive and catalase-negative rods showing positive results after the well diffusion assay were subjected to phenotypic identification by API 50 CHL System (bioMérieux, Lyon, France). The API test was preceded by assays, which considered growth at 15 and 45°C, CO_2 production from glucose, NH_3 production from arginine and esculine hydrolysis.

All bacteriocin-producer strains were also identified by 16S rDNA sequencing. Genomic DNA of the isolates was extracted from 2 ml of overnight cultures grown in MRS by the method of De Los Reyes-Gavilàn *et al.* (1992). Primers (MWG Biotech AG, Ebersberg, Germany), designed from conserved sequences within bacterial 16S rRNA region, were LacbF 5′-TGCCTAATACATGCAAGT-3′ and LacbR 5′-CTTGTTACGACTTCACCC-3′ derived from the homologous regions of several 16S rRNA sequences of *Lactobacillus* species, and corresponding to positions 38 to 55, and 1503 to 1520, respectively, of 16S rRNA region from *Lb. brevis* (accession no. AF515220).

PCR was carried out using *Taq* PCR Master Mix Kit (Qiagen, Hilden, Germany). The 50 μl PCR mixtures contained 25 μl of *Taq* PCR Master Mix, 2 pmol of each primer, 2 μl of template DNA (*ca* 50 ng of DNA) and sterile distilled H_2O. PCR amplification of the 16S rDNA was performed using the Primus PCR System (MWG Biotech AG). The PCR program comprised an initial template denaturation step for 2 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 49°C and extension for 1 min at 72°C. The final extension step was for 7 min at 72°C.

The PCR products were purified by the ConcertTM Rapid PCR Purification System (Gibco BRL, Cergy Pontoise, France). DNA sequencing reactions were performed by MWG Biotech AG.

Effect of enzymes and heat treatment on the antimicrobial activity

Sensitivity to proteolytic enzymes of each supernatant containing antimicrobial activity was tested by treatment

Table 1 Inhibitory activity of sourdough lactobacilli determined by well diffusion assay

Indicator strains	Media	Producer strains*				
		4DE	CS1	8CF	2MF8	3DM
Lactobacillus farciminis CC10	SDB	3·2 ± 0·3†	2·8 ± 0·3	1·3 ± 0·3	–	–
Lactobacillus farciminis 5C1	SDB	–	1·0 ± 0·0	–	2·8 ± 0·3	–
Lactobacillus farciminis 2XA6	SDB	–	–	–	–	–
Lactobacillus farciminis 9XC8	SDB	–	–	–	–	–
Lactobacillus sakei LMG 2313	mMRS	2·7 ± 0·3	2·8 ± 0·3	2·8 ± 0·3	3·0 ± 0·0	2·3 ± 0·3
Lactobacillus delbrueckii subsp. *bulgaricus* B397	MRS	–	–	–	–	–
Lactobacillus acidophilus DSM 20079	MRS	–	–	–	–	–
Lactobacillus pontis 30	MRS	–	–	–	–	–
Lactobacillus pontis 37	MRS	–	3·0 ± 0·0	–	2·7 ± 0·3	–
Lactobacillus casei ssp. *pseudoplantarum* 109V	MRS	–	–	–	–	–
Lactobacillus casei ssp. *casei* B44	MRS	–	–	–	–	–
Lactobacillus curvatus 1HD	MRS	–	–	–	–	–
Lactobacillus amylovorus ATCC 33620	MRS	–	–	–	–	–
Lactobacillus rhamnosus ATCC 7469	MRS	–	–	–	–	–
Lactobacillus zeae ATCC 393	MRS	–	–	–	–	–
Lactobacillus paracasei ssp. *paracasei* ATCC 25302	MRS	–	–	–	–	–
Lactobacillus viridescens ATCC 12706	MRS	–	–	–	–	–
Lactobacillus alimentarius DA 70	mMRS	–	–	–	–	–
Lactobacillus alimentarius AN2	mMRS	–	–	–	–	–
Lactobacillus alimentarius F13	mMRS	–	–	–	–	–
Lactobacillus alimentarius O9	mMRS	–	–	–	–	–
Lactobacillus alimentarius 5α	mMRS	–	–	–	–	–
Lactobacillus alimentarius 3D	mMRS	–	–	–	–	–
Lactobacillus alimentarius 15β	mMRS	–	3·2 ± 0·3	–	3·3 ± 0·3	–
Lactobacillus sanfranciscensis CB1	SDB	–	–	–	–	–
Lactobacillus sanfranciscensis A22	SDB	–	–	–	2·0 ± 0·0	–
Lactobacillus sanfranciscensis 9N	SDB	–	–	–	–	–
Lactobacillus sanfranciscensis 7H	SDB	–	–	–	–	–
Lactobacillus sanfranciscensis 13R	SDB	–	–	–	–	–
Lactobacillus sanfranciscensis I1	SDB	1·5 ± 0·0	–	–	–	–
Lactobacillus brevis AM8	SDB	–	–	–	–	–
Lactobacillus brevis AM7	SDB	–	–	–	–	–
Lactobacillus brevis 1Q	SDB	–	–	–	–	–
Lactobacillus brevis I5	SDB	–	–	–	–	–
Lactobacillus brevis DA64	SDB	–	–	–	–	–
Lactobacillus brevis 10R	SDB	–	–	–	–	–
Lactobacillus fermentum I2	SDB	–	–	1·2 ± 0·3	1·3 ± 0·3	–
Lactobacillus fermentum CD5	SDB	–	1·8 ± 0·3	–	2·5 ± 0·0	–
Lactobacillus plantarum 3DM	SDB	–	–	–	–	–
Lactobacillus plantarum 20	SDB	–	–	–	1·0 ± 0·0	–
Lactobacillus plantarum AD4	SDB	–	–	–	–	–
Lactobacillus plantarum CF1	SDB	1·5 ± 0·5	3·0 ± 0·0	–	2·5 ± 0·5	–
Lactobacillus plantarum 13	SDB	–	3·0 ± 0·5	–	3·3 ± 0·3	–
Lactobacillus plantarum 4DE	SDB	–	–	–	–	–
Lactobacillus fructivorans DA110	SDB	–	–	–	–	–
Lactobacillus fructivorans P4	SDB	–	1·0 ± 0·0	–	1·0 ± 0·0	–
Lactobacillus fructivorans DD7	SDB	–	1·0 ± 0·0	–	2·7 ± 0·3	–
Lactobacillus hilgardii 51B	mMRS	–	–	1·3 ± 0·3	2·0 ± 0·0	–
Lactobacillus hilgardii 25Y	mMRS	–	–	–	–	–
Leuconostoc spp.	MRS	1·5 ± 0·5	1·5 ± 0·3	–	–	–
Listeria innocua 4202	TSYE	–	–	–	–	–

Table 1 (*Contd.*)

Indicator strains	Media	Producer strains*				
		4DE	CS1	8CF	2MF8	3DM
Streptococcus thermophilus X6	M17	–	–	–	–	–
Weissella confusa DSM 20196	MRS	–	–	–	–	–
Bacillus cereus 660	BN	–	–	–	–	–
Bacillus subtilis 813	BN	–	–	–	–	–
Saccharomyces cerevisiae 141	WL	–	–	–	–	–
Saccharomyces exiguus M14	WL	–	–	–	–	–

*Producer strains are termed by the collection reference abbreviation: 4DE, *Lb. plantarum*; CS1, *Lactobacillus* spp.; 8CF and 2MF8, *Lb. pentosus*; 3DM, *Lb. plantarum*.

†Width of the inhibition zone (mm).

'–', No inhibition zone. Results indicate mean ± S.D. of three independent experiments.

with proteinase K (12·5 U mg^{-1}), protease B (45 U mg^{-1}) and trypsin (10·6 U mg^{-1}) at a final concentration of 1 mg ml^{-1} in phosphate buffer (pH 7·0). The supernatants were examined for the sensitivity to lipolityc and amylolityc enzymes, by lipase (50 U mg^{-1} and α-amylase (15 U mg^{-1}), respectively, at a final concentration of 1 mg ml^{-1} in phosphate buffer (pH 7·0). The supernatants were incubated with these enzymes at 37°C for 2 h after which remaining activity was determined by a well diffusion assay.

Sensitivity to high temperature was determined by heat treatment of the supernatants at 100°C for 20 min, 100°C for 60 min and by sterilization (121°C for 15 min).

Stability of the antimicrobial activity at different pH, refrigerated storage and organic solvents

Bacteriocin-containing cell-free supernatants were adjusted to varying pH values, ranging between 3·0 and 11·0, with 5 mol l^{-1} HCl or 1 mol l^{-1} NaOH, incubated at 28°C for 1 h and assayed for residual activity. To evaluate the stability over time, antimicrobial activity-containing supernatant was stored at −20 and 4°C for 60 days and activity was evaluated every 10 days. The effect of organic solvents was evaluated after concentration and treatment of active supernatants with 5, 10, and 15% ethanol.

Mode of action

To study the effect of antimicrobial compounds on sensitive cells, 2 ml of a 10-fold concentrated (under vacuum), adjusted to pH 6·5 and catalase treated, supernatant was added to 18 ml of mMRS in which, after filtration through a 0·22-μm pore size filter (Nucleopore, Costar Corporation), the indicator strain *Lb. sakei* LMG 2313 was inoculated at a cellular concentration of approx. 10^3 CFU ml^{-1}. As a negative control, the supernatant of *Lb. sanfranciscensis* 7H (a strain which does not produce any antimicrobial compound), similarly treated as described above, was used. Cells were cultivated at 30°C for 24 h and at 2-h intervals, the optical density at 620 nm ($O.D._{620}$) and the number of viable *Lb. sakei* LMG 2313 cells on mMRS agar plates was determined.

Adsorption studies

The effect of the pH on adsorption of bacteriocin onto producer cells was evaluated as reported by Yang *et al.* (1992) and Todorov *et al.* (1999). After 18 h of growth, the culture broth was adjusted to pH 6·0 and incubated at 4°C for 30 min. After centrifugation, cell-free supernatant was assayed for bacteriocin activity not adsorbed onto the cells while the cells were washed with sodium phosphate buffer, pH 6·5, centrifuged, resuspended in 0·1 mol l^{-1} NaCl (pH 2·0) (adjusted with 5% phosphoric acid) and mixed with a magnetic stirrer for 1 h at 4°C. Cell suspension was centrifuged (4500 *g* for 30 min) and the resulting supernatant was neutralized and tested for antibacterial activity released by the cells after treatment at pH 2·0.

Production study

In order to determine optimal conditions for bacteriocin production, tests were performed to evaluate the effect of culture medium, temperature, time of incubation and pH. Cells of the producer strain were cultivated at 15, 30, 37 and 42°C for 48 h in mMRS, MRS, SDB and a synthetic modified Amino Acid Assay Medium (AAAM) (Difco) modified as follows: ammonium chloride and sodium chloride were omitted. Other modifications concerned the reduction of dextrose (5 g l^{-1} instead of 50 g l^{-1}), sodium acetate (2·5 g l^{-1} instead of 40 g l^{-1}) and the addition of diammonium citrate (1 g l^{-1}), Tween-80 (0·5 g l^{-1}) and maltose (5 g l^{-1}). The final pH of the medium was 6·0.

After selection of the optimal medium and growth conditions, the influence of a constant pH during the growth was evaluated by maintaining a stable pH of 6·0 by the periodic addition of 6 mol l^{-1} NaOH. Antimicrobial activity contained in the cell-free supernatant was evaluated each 3 h by a well diffusion assay as described above.

Sourdough production and evaluation of *in situ* antimicrobial activity

Lactobacilli and lactococci used as sourdough starters were cultured in SDB (Kline and Sugihara 1971) or mMRS, and M17 (Difco) broth, respectively, at 30°C for 24 h. Cells were harvested by centrifugation at 6500 *g* for 10 min, washed twice with sterile, distilled water, and then resuspended in sterile distilled water; the cell suspension, diluted 1 : 10, gave an $O.D._{620}$ of 0·25. Wheat flour, containing 12·5% moisture; protein (N × 5·70) 11% of dry matter (DM); fat 1·82% of DM; and ash, 0·60% of DM. Wheat flour (50 g), tap water (22·4 ml), and cellular suspension (7·6 ml) of one or more micro-organisms, was used to produce doughs with a dough yield (weight of the dough/weight of the flour × 100) of 160 and with a cellular concentration of about 10^4 CFU g^{-1}. Doughs (80 g) were individually placed in glass beaker and incubated at 30°C for 21 h. A reference dough (R) without starter was produced and incubated as above described. In case the bacteriocin-containing supernatant was added to the dough instead of the producer strain, an equivalent volume of water (30 ml) was removed in order to obtain dough with the same dough yield.

Extraction of antimicrobial activity from sourdough

At the end of fermentation (30°C for 21 h), 10 g of each dough was suspended in 90 ml of a 40% acetonitrile – 0·1% (v/v) trifluoroacetic acid solution (on account of the hydrophobic nature of the inhibitory substances), homogenized, centrifuged at 15 000 *g* for 10 min, freeze-dried and then resuspended in 4 ml of a 50% (v/v) ethanol solution. The suspension extracted from a dough of flour and water, without the addition of starter LAB and incubated as above reported, was assayed in order to avoid false positive results due to the putative inhibitory activity produced by micro-organisms spontaneously growing in the dough. All suspensions were subjected to a well diffusion assay.

Sourdough characteristics

Sourdoughs were analysed immediately after mixing and after 6 and 21 h of fermentation. Depending on the LAB added as starters, plate counts were performed using MRS, SDB, MRS containing sucrose instead of glucose (final pH 5·0) (MRSs) or M17 media, at 30°C for 72 h. The presence of micro-organisms added as starter was also confirmed by microscopic analysis and, after culture isolation and purification, by phenotypic identification with API 50 CHL System (bioMérieux). The pH was determined by a Corning pH-Meter (Corning, Halstead, Essex, UK) on the diluted (10 g of dough and 90 ml of distilled water) and homogenized (Classic Blender; PBI International, Milan, Italy) sourdoughs.

Statistical analyses

All the experiments, with the exception of dough fermentations (performed in duplicate) were carried out in three independent experiments and the results are shown as mean ± S.D.

RESULTS

Screening of sourdough *Lactobacillus* spp. for antimicrobial compound production

A total of 437 *Lactobacillus* strains, isolated from 70 sourdoughs, were initially screened for antimicrobial compound production against four indicator strains (*Lb. farciminis* CC10, *Lb. sakei* LMG 2313, *Lb. delbrueckii* ssp. *bulgaricus* B397, and *Listeria innocua* 4202) by means of the agar-spot deferred method. In this step the possible inhibitory effect of the organic acids and of hydrogen peroxide was not excluded. Eighty-five of the 437 strains tested produced an inhibition zone against one or more indicators. Subsequently, the cell-free supernatants from the 85 strains were treated with catalase, neutralized, sterilized by filtration and tested by the well diffusion assay against the same four indicators. Only five unidentified lactobacilli (*Lactobacillus* spp. 3DM, 4DE, 8CF, 2MF8 and CS1) were found to maintain the antimicrobial activity against some indicators, showing a measurable clear zone around the well. To better evaluate the inhibitory activity of the five producer strains, their cell-free supernatants were tested against other 53 indicators (Table 1). All antimicrobial activities tested were active against *Lb. sakei* LMG 2313 while the inhibition of other lactobacilli was dependent on the producer strain. *Lactobacillus* spp. 2MF8 and CS1 showed the largest spectra (13 and 11 strains of 57 were inhibited, respectively). *Leuconostoc* spp. were inhibited only by *Lactobacillus* spp. 4DE and CS1, while many of the other micro-organisms used as indicators (*Listeria*, *Streptococcus*, *Weissella*, *Bacillus*, and *Saccharomyces* spp.) were not inhibited by any producer strains.

The above five lactobacilli, not identified previously, were subjected to phenotypic identification and 16S rDNA

sequencing, and were thus classified as *Lb. pentosus* 2MF8, *Lb. pentosus* 8CF, *Lb. plantarum* 3DM, and *Lb. plantarum* 4DE. Strain CS1 remained unclassified as it probably belongs to a new *Lactobacillus* spp. Work is in progress to further classify this strain.

Effect of enzymes and heat treatments

The cell-free supernatants from the five strains producing antimicrobial substances were assayed for sensitivity to hydrolytic enzymes and heat. All antibacterial compounds produced by those strains were inactivated by proteolytic enzymes (Table 2), indicating that the inhibitory compounds are of proteinaceus nature, a general characteristic of bacteriocins. As protein compounds inhibitory to closely related bacteria can be included in the category of the bacteriocins (Tagg *et al.* 1976; Jack *et al.* 1995) and because the substances of this study have not yet been characterized for amino acid and nucleotide sequences, they will be referred to as BLIS. The cell-free supernatants derived from strains *Lb. plantarum* 4DE and 3DM, and *Lactobacillus* spp . CS1, were insensitive to α-amylase, whereas those derived from *Lb. pentosus* 8CF and 2MF8 were sensitive. Similarly, the supernatant activity from *Lb. plantarum* 4DE and *Lactobacillus* spp. CS1 was not inhibited by lipase, whereas that from *Lb. pentosus* 8CF and 2MF8, and *Lb. plantarum* 3DM was inhibited. Moreover, the supernatants from strains CS1, 8CF and 2MF8 were resistant to treatment of

Table 2 Effect of enzymes, heat treatment, pH, storage, and organic solvent on inhibitory activity of sourdough lactobacilli determined by well diffusion assay*

	Producer strains†				
Treatment	4DE	CS1	8CF	2MF8	3DM
Control (supernatant not treated)	2·5 ± 0·5‡	2·8 ± 0·3	2·3 ± 0·3	3·0 ± 0·5	2·2 ± 0·3
Enzymes					
Proteinase K	–	–	–	–	–
Protease B	–	–	–	–	–
Trypsin	–	–	–	–	–
α-Amylase	2·5 ± 0·5	2·7 ± 0·3	–	–	2·3 ± 0·3
Lipase	2·5 ± 0·0	2·7 ± 0·3	–	–	–
Heat-treatment					
100°C for 20 min	1·5 ± 0·0	2·3 ± 0·3	2·0 ± 0·0	2·5 ± 0·5	–
100°C for 60 min	–	2·2 ± 0·3	2·0 ± 0·0	2·3 ± 0·3	–
Sterilization (121 °C for 15 min)	–	1·7 ± 0·3	1·5 ± 0·5	1·8 ± 0·3	–
pH					
3	1·5 ± 0·0	2·3 ± 0·3	2·0 ± 0·0	2·8 ± 0·3	1·8 ± 0·3
4	1·7 ± 0·3	2·5 ± 0·0	2·2 ± 0·3	2·8 ± 0·3	1·8 ± 0·3
5	2·5 ± 0·5	2·8 ± 0·3	2·3 ± 0·3	3·0 ± 0·5	2·3 ± 0·3
6	2·5 ± 0·5	2·8 ± 0·3	2·3 ± 0·3	3·0 ± 0·5	2·3 ± 0·3
7	2·5 ± 0·5	2·8 ± 0·3	2·3 ± 0·3	3·0 ± 0·5	2·3 ± 0·3
8	1·8 ± 0·3	2·5 ± 0·5	2·0 ± .05	2·8 ± 0·3	1·8 ± 0·3
9	1·7 ± 0·3	1·8 ± 0·3	1·5 ± 0·5	1·8 ± 0·3	–
10	–	1·8 ± 0·3	–	1·8 ± 0·3	–
11	–	–	–	–	–
Storage for 90 days					
−20°C	2·3 ± 0·3	2·5 ± 0·5	2·2 ± 0·3	2·8 ± 0·3	2·3 ± 0·3
4°C	1·8 ± 0·3	2·2 ± 0·3	1·8 ± 0·3	2·8 ± 0·3	1·5 ± 0·5
25°C	–	1·8 ± 0·3	–	–	–
Organic solvent					
C_2H_5OH 5%	1·7 ± 0·3	2·8 ± 0·3	2·3 ± 0·3	3·0 ± 0·5	1·7 ± 0·3
C_2H_5OH 10%	1·7 ± 0·3	2·5 ± 0·5	2·3 ± 0·3	2·8 ± 0·3	1·5 ± 0·5
C_2H_5OH 15%	1·7 ± 0·3	2·5 ± 0·5	2·2 ± 0·3	2·8 ± 0·3	1·5 ± 0·5

*All assays were conducted with *Lb. sakei* LMG 2313 as indicator strain.

†Producer strains are termed by the collection reference abbreviation: 4DE, *Lb. plantarum*; CS1, *Lactobacillus*spp.; 8CF and 2MF8, *Lb. pentosus*; 3DM, *Lb. plantarum*.

‡Width of the inhibition zone (mm).

'–', No inhibition zone. Results indicate mean ± S.D. of three independent experiments.

100°C for 60 min, and were partially inactivated by sterilization, while the activity contained in the cell-free supernatants from *Lb. plantarum* 4DE and 3DM were partially or totally lost, respectively, after heat treatment at 100°C for 20 min (Table 2).

Stability of the antimicrobial activity at different pH values, refrigerated storage and after treatments with an organic solvent

Stability of antimicrobial activity was evaluated under various conditions. All samples retained full activity in the pH range 5–7 (Table 2). Cell-free supernatant from *Lactobacillus* spp. CS1, *Lb. pentosus* 2MF8 and *Lb. pentosus* 8CF remained fully active at pH values ranging from 3 to 8 while a reduction of activity was observed above such value. The strains *Lb. plantarum* 4DE and 3DM showed a reduction of activity also at pH values of 3–4 and 8–9 (Table 2).

The activity of *Lactobacillus* spp. CS1 was stable during frozen (−20°C) and refrigerated (+4°C) storage for at least 3 months and partially lost when stored at 25°C. The inhibitory activities produced by the other strains were completely lost after 3 months at 25°C and retained the maximum activity only when stored at −20°C (Table 2). The antimicrobial activity of supernatants from *Lactobacillus* spp. CS1, *Lb. pentosus* 8CF and *Lb. pentosus* 2MF8 was completely retained in the presence of 15% or less ethanol, while the *Lb. plantarum* 4DE and 3DM activities were reduced by ethanol at concentrations of 5% and higher.

Mode of action of the antibacterial substances

The addition of cell-free supernatant of *Lb. pentosus* 2MF8 to 1×10^3 CFU ml^{-1} cells of the indicator strain *Lb. sakei* LMG 2313 arrested cell growth for the first 10 h after which growth resumed, ultimately reaching a cell density of $2{\cdot}6 \times 10^6$ CFU ml^{-1} after 24 h. In contrast, in the presence of a cell-free supernatant from a negative control strain (nonproducer), the indicator strain showed a normal growth reaching 1×10^6 CFU ml^{-1} after 10 h and $1{\cdot}8 \times 10^9$ CFU ml^{-1} at 24 h. The other four producer strains displayed a similar inhibition of the indicator.

Adsorption studies

Yang *et al.* (1992) reported that 93–100% of the bacteriocin is adsorbed to produce cells at pH 6·0 while <5% adsorption took place at pH 1·5–2·0.

Under the conditions tested the activity of cell-free supernatant of the five producer strains obtained after treatment of cells at pH 2·0 was very low indicating that the inhibitory compound is completely released in the medium during growth and no significant absorption takes place following adjustment of the pH of the broth to 6·0 before harvesting the cells by centrifugation.

Production studies

The maximum activity of the BLIS produced by the five sourdough strains, as evaluated by the critical dilution assay against *Lb. sakei* LMG 2313, was obtained in MRS after 12–22 h of incubation at 30°C (values of 40 and 160 AU ml^{-1} for *Lb. plantarum* 4DE and 3DM, respectively, and of 320 AU ml^{-1} for *Lb. pentosus* and *Lactobacillus* spp. CS1). BLIS production, growth and pH kinetics for *Lb. pentosus* 2MF8 are shown in Fig. 1.

After 22 h of growth at 30°C or after 18 h at 37°C the activity decreased and higher reductions were observed at higher/lower temperature (data not shown). Maintaining a constant pH of 6·0 during growth did not improve the inhibitory activity. Similar results were obtained, only for *Lactobacillus* spp. CS1, using the synthetic mAAAM (Difco), not containing complex nitrogen sources, instead of MRS while no inhibitory activity was detected in mAAAM medium after growth of the other producer strains.

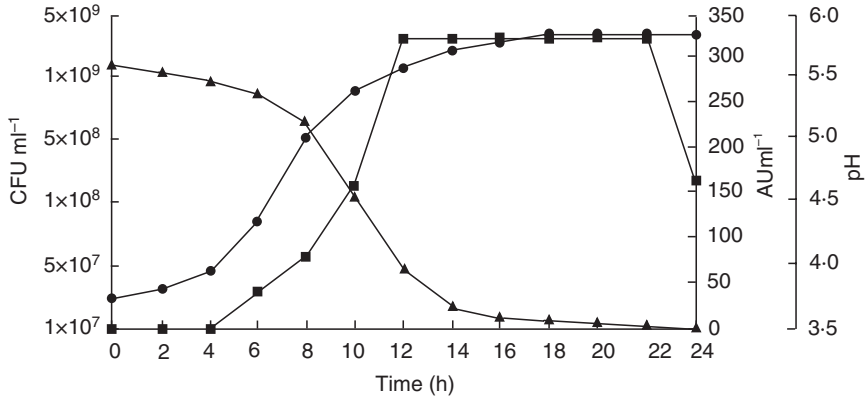

Fig. 1 *Lactobacillus pentosus* 2MF8 growth and BLIS production at uncontrolled pH. Colony-forming units (CFU ml^{-1}), ●; BLIS titre (AU ml^{-1}), ■; pH, ▲

Evaluation of *Lc. lactis* activity against sourdough LAB

In order to compare the antibacterial spectrum of sourdough LAB with that of bacteriocin-producer strains isolated from other foods, *Lc. lactis* M30 and M36, isolated from raw, unmalted barley (Hartnett *et al.* 2002), were evaluated against *Lb. sakei* LMG 2313, *Lb. sanfranciscensis* (six strains), *Lb. plantarum* (six strains), *B. subtilis* 813, and *B. cereus* 660 (Table 3). *Lactococcus lactis* M30 and M36 were characterized by an almost identical spectrum of activity. They inhibited 12 and 13 indicator strains of 15 tested, respectively, and showed no activity vs *Lb.sanfranciscensis* CB1 and 7H (Table 3). Antimicrobial compounds from lactococci showed an inhibition of 800 AU ml^{-1} against *Lb. sakei* LMG2313 and were also characterized by an high antilisterial activity (25 600 AU ml^{-1}) (Hartnett *et al.* 2002).

Evaluation of the *in situ* antibacterial activity

In order to evaluate the capability of the BLIS-producing LAB to inhibit the indicator strains *in situ*, *Lc. lactis* M30 and *Lb. pentosus* 2MF8 (BLIS producers); *Lc. lactis* Q13 and *Lb. plantarum* 20 (negative controls); *Lb. sakei* LMG 2313, *Lb. plantarum* 20 and *Lb. sanfranciscensis* CB1 (indicator strains) were preliminarily used as individual starter to produce sourdoughs. Starting at a pH of 5·98–6·04, the six sourdoughs fermented with individual strains reached a pH of 4·07–4·82 after 21 h of fermentation (doughs A–F, Table 4). *Lactobacillus sakei* LMG 2313, *Lc. lactis* M30 and *Lc. lactis* Q13 were the LAB exhibiting the lowest acidifying ability. All six strains grew in the dough from 2·6–$5·9 \times 10^4$ to $2·9 \times 10^8$–$2·6 \times 10^9$ CFU g^{-1} after 21 h of fermentation (Table 4). During the same time period, mesophilic LAB reached a cell density of $3·7 \times 10^6$ CFU g^{-1} in the reference dough produced without starter (dough R, Table 4).

Table 3 Inhibitory activity of selected bacteriocin-producing *Lactococcus lactis* determined by well diffusion assay

	Producer strains*	
Indicator strains	M30	M36
Lb. sakei LMG 2313	6·5 ± 0·5†	6·5 ± 0·5
Lb. sanfranciscensis CB1	–	–
Lb. sanfranciscensis A22	3·0 ± 0·5	2·8 ± 0·3
Lb. sanfranciscensis 9N	2·8 ± 0·3	2·7 ± 0·3
Lb. sanfranciscensis 7H	–	–
Lb. sanfranciscensis 13R	3·0 ± 0·5	2·8 ± 0·3
Lb. sanfranciscensis I1	2·8 ± 0·3	2·8 ± 0·3
Lb. plantarum 3DM	1·8 ± 0·3	2·0 ± 0·0
Lb. plantarum 20	6·0 ± 0·5	6·0 ± 0·5
Lb. plantarum AD4	1·8 ± 0·3	1·8 ± 0·3
Lb. plantarum CF1	3·3 ± 0·3	3·0 ± 0·5
Lb. plantarum 13	1·8 ± 0·3	1·5 ± 0·5
Lb. plantarum 4DE	1·8 ± 0·3	1·5 ± 0·5
B. subtilis 813	6·2 ± 0·3	5·8 ± 0·3
B. cereus 660	–	4·2 ± 0·3

*Producer strains are termed by the collection reference abbreviation: M30–M36, *Lc. lactis*. The strain M30 is a BLIS producer.
†Width of the inhibition zone (mm).
'–', No inhibition zone. Results indicate mean ± S.D. of three independent experiments.

The results of the sourdough fermentation obtained by mixed starters are also reported in Table 4. A selective growth of *Lb. sakei* LMG 2313, *Lb. plantarum* 20, and *Lb. pentosus* 2MF8 was possible using the MRSs. This medium was useful to easily distinguish the above strains from *Lc. lactis* or from *Lb. sanfranciscensis* after associate growth. In the sourdoughs started with the associations *Lb. pentosus* 2MF8–*Lb. sakei* LMG 2313 (dough no. 1) or *Lc. lactis* M30–*Lb. sakei* LMG 2313 (dough no. 2) the BLIS producers showed a final cell concentration similar to that obtained at the end of individual fermentation ($4·4 \times 10^9$ vs. $2·6 \times 10^9$ CFU g^{-1} and $1·4 \times 10^9$ vs. $1·5 \times 10^9$ CFU g^{-1}, respectively). In contrast, in the presence of *Lb. pentosus* 2MF8 (dough no. 1) the indicator strain *Lb. sakei* LMG 2313 showed a decrease with respect to the individual fermentation (6×10^7 vs. $2·9 \times 10^8$ CFU g^{-1}).

In the presence of *Lc. lactis* M30 (dough no. 2) the indicator strain *Lb. sakei* LMG 2313 appears to reduce its growth, i.e. after 21 h of fermentation the cellular concentration of this organism does not exceed $4·0 \times 10^6$ CFU g^{-1}. The *in situ* inhibitory activity of *Lc. lactis* M30 was also obvious against *Lb. plantarum* 20, which discontinued growth at a cell density of $4·1 \times 10^6$ CFU g^{-1} at the end of fermentation (dough no. 3). In both cases the indicator strains were not easily distinguishable from contaminating sourdough LAB, which reached similar concentration (dough R). Both indicator strains exhibited normal growth, similar to that obtained during individual fermentations, when associated with the negative control strains *Lb. plantarum* 20 or *Lc. lactis* Q13 (dough nos. 4 and 5).

In order to exclude the possibility that the observed inhibition was dependent on substrate competition, the active, neutralized and catalase-treated cell-free supernatant from the *Lb. pentosus* 2MF8 or *Lc. lactis* M30, instead of viable cells of the same strains, was added to the doughs fermented with *Lb. sakei* LMG 2313 or *Lb. plantarum* 20, respectively (dough nos. 9 and 10). Also in these cases the indicator strains grew only to a cell density not exceeding $3·2 \times 10^6$ and 5×10^6 CFU g^{-1}, respectively, while the final pH of the dough showed values similar to the reference dough produced without starters (dough R).

With the aim to evaluate the possible contribution of the antibacterial compound producer strains on the implantation

Table 4 Kinetics of growth and acidification of selected lactobacilli and lactococci during individual (A–F) and mixed (1–10) sourdough fermentation

Dough	Starters	Culture media*	CFU g^{-1} T.O.	pH T.O.	CFU g^{-1} 6 h	pH 6 h	CFU g^{-1} 21 h	pH 21 h
R†	Without starter	MRS	$8{\cdot}0 \times 10^{2} \pm 0{\cdot}42$‡	6·02 ± 0·03	$2{\cdot}0 \times 10^{3} \pm 0{\cdot}92$‡	5·95 ± 0·03	$3{\cdot}7 \times 10^{6} \pm 1{\cdot}17$‡	5·80 ± 0·04
A	*Lb. sakei* LMG 2313	MRSs	$2{\cdot}6 \times 10^{4} \pm 0{\cdot}92$	6·04 ± 0·03	$3{\cdot}2 \times 10^{4} \pm 0{\cdot}71$	6·03 ± 0·04	$2{\cdot}9 \times 10^{8} \pm 1{\cdot}27$	4·82 ± 0·03
B	*Lc. lactis* M30	M17	$5{\cdot}9 \times 10^{4} \pm 0{\cdot}99$	6·03 ± 0·05	$3{\cdot}6 \times 10^{7} \pm 1{\cdot}69$	5·81 ± 0·04	$1{\cdot}5 \times 10^{9} \pm 1{\cdot}06$	4·53 ± 0·05
C	*Lb. pentosus* 2MF8	MRSs	$3{\cdot}9 \times 10^{4} \pm 0{\cdot}71$	6·03 ± 0·03	$5{\cdot}3 \times 10^{5} \pm 1{\cdot}41$	6·00 ± 0·04	$2{\cdot}6 \times 10^{9} \pm 1{\cdot}69$	4·09 ± 0·05
D	*Lb. plantarum* 20	MRSs	$2{\cdot}8 \times 10^{4} \pm 0{\cdot}56$	6·02 ± 0·04	$3{\cdot}9 \times 10^{4} \pm 1{\cdot}77$	6·02 ± 0·03	$2{\cdot}6 \times 10^{9} \pm 1{\cdot}77$	4·16 ± 0·04
E	*Lc. lactis* Q13	M17	$4{\cdot}7 \times 10^{4} \pm 0{\cdot}21$	5·98 ± 0·03	$5{\cdot}2 \times 10^{6} \pm 1{\cdot}20$	5·94 ± 0·05	$9{\cdot}2 \times 10^{8} \pm 1{\cdot}56$	4·59 ± 0·05
F	*Lb. sanfranciscensis* CB1	SDB	$3{\cdot}3 \times 10^{4} \pm 0{\cdot}92$	6·03 ± 0·03	$7{\cdot}9 \times 10^{4} \pm 1{\cdot}62$	6·00 ± 0·05	$2{\cdot}1 \times 10^{9} \pm 1{\cdot}38$	4·07 ± 0·05
1	*Lb. pentosus* 2MF8	MRSs	$6{\cdot}8 \times 10^{4} \pm 0{\cdot}71$	6·02 ± 0·05	$8{\cdot}7 \times 10^{5} \pm 1{\cdot}27$	6·03 ± 0·03	$4{\cdot}4 \times 10^{9} \pm 1{\cdot}20$	3·95 ± 0·05
	Lb. sakei LMG 2313	MRSs	$2{\cdot}3 \times 10^{4} \pm 0{\cdot}23$		$2{\cdot}0 \times 10^{4} \pm 0{\cdot}71$		$6{\cdot}0 \times 10^{7} \pm 0{\cdot}42$	
2	*Lc. lactis* M30	M17	$5{\cdot}1 \times 10^{4} \pm 1{\cdot}20$	6·03 ± 0·04	$3{\cdot}2 \times 10^{7} \pm 1{\cdot}55$	5·87 ± 0·04	$1{\cdot}4 \times 10^{9} \pm 0{\cdot}92$	4·51 ± 0·03
	Lb. sakei LMG 2313	MRSs	$2{\cdot}6 \times 10^{4} \pm 0{\cdot}71$		$2{\cdot}8 \times 10^{4} \pm 1{\cdot}27$		$4{\cdot}0 \times 10^{6} \pm 0{\cdot}99$§	
3	*Lc. lactis* M30	M17	$8{\cdot}2 \times 10^{4} \pm 0{\cdot}78$	6·05 ± 0·04	$1{\cdot}2 \times 10^{7} \pm 1{\cdot}41$	5·75 ± 0·04	$2{\cdot}1 \times 10^{9} \pm 1{\cdot}41$	4·57 ± 0·05
	Lb. plantarum 20	MRSs	$4{\cdot}8 \times 10^{4} \pm 1{\cdot}27$		$5{\cdot}0 \times 10^{4} \pm 1{\cdot}41$		$4{\cdot}1 \times 10^{6} \pm 1{\cdot}69$§	
4	*Lb. plantarum* 20	MRSs	$4{\cdot}3 \times 10^{4} \pm 0{\cdot}64$	6·06 ± 0·04	$2{\cdot}3 \times 10^{5} \pm 0{\cdot}99$	6·02 ± 0·05	$5{\cdot}6 \times 10^{8} \pm 1{\cdot}27$	5·02 ± 0·05
	Lb. sakei LMG 2313	MRSs	$2{\cdot}8 \times 10^{4} \pm 0{\cdot}78$		$7{\cdot}8 \times 10^{4} \pm 0{\cdot}78$		$1{\cdot}8 \times 10^{8} \pm 1{\cdot}55$	
5	*Lc. lactis* Q13	M17	$6{\cdot}9 \times 10^{4} \pm 1{\cdot}20$	6·05 ± 0·03	$4{\cdot}1 \times 10^{6} \pm 1{\cdot}69$	6·03 ± 0·03	$7{\cdot}4 \times 10^{8} \pm 1{\cdot}20$	4·70 ± 0·04
	Lb. plantarum 20	MRSs	$4{\cdot}2 \times 10^{4} \pm 0{\cdot}56$		$2{\cdot}1 \times 10^{5} \pm 0{\cdot}64$		$2{\cdot}1 \times 10^{8} \pm 1{\cdot}41$	
6	*Lb. plantarum* 20	MRSs	$2{\cdot}7 \times 10^{4} \pm 0{\cdot}78$	6·05 ± 0·03	$7{\cdot}9 \times 10^{4} \pm 1{\cdot}06$	6·02 ± 0·04	$9{\cdot}4 \times 10^{8} \pm 1{\cdot}69$	4·13 ± 0·05
	Lb. sanfranciscensis CB1	SDB	$3{\cdot}5 \times 10^{4} \pm 1{\cdot}77$		$9{\cdot}3 \times 10^{4} \pm 0{\cdot}64$		$3{\cdot}2 \times 10^{9} \pm 1{\cdot}06$	
7	*Lc. lactis* M30	M17	$7{\cdot}3 \times 10^{4} \pm 1{\cdot}20$	6·04 ± 0·05	$9{\cdot}7 \times 10^{6} \pm 0{\cdot}99$	5·84 ± 0·04	$1{\cdot}9 \times 10^{9} \pm 1{\cdot}55$	4·32 ± 0·05
	Lb. plantarum 20	MRSs	$3{\cdot}2 \times 10^{4} \pm 1{\cdot}41$		$3{\cdot}8 \times 10^{4} \pm 1{\cdot}77$		$4{\cdot}2 \times 10^{6} \pm 1{\cdot}38$§	
	Lb. sanfranciscensis CB1	SDB	$3{\cdot}9 \times 10^{4} \pm 1{\cdot}55$		$8{\cdot}3 \times 10^{4} \pm 1{\cdot}20$		$1{\cdot}7 \times 10^{9} \pm 1{\cdot}27$	
8	*Lc. lactis* Q13	M17	$5{\cdot}9 \times 10^{4} \pm 0{\cdot}57$	6·05 ± 0·03	$8{\cdot}5 \times 10^{6} \pm 0{\cdot}64$	6·03 ± 0·03	$8{\cdot}2 \times 10^{8} \pm 1{\cdot}27$	4·28 ± 0·04
	Lb. plantarum 20	MRSs	$3{\cdot}5 \times 10^{4} \pm 1{\cdot}06$		$6{\cdot}7 \times 10^{4} \pm 0{\cdot}78$		$3{\cdot}5 \times 10^{8} \pm 0{\cdot}64$	
	Lb. sanfranciscensis CB1	SDB	$3{\cdot}4 \times 10^{4} \pm 0{\cdot}78$		$9{\cdot}1 \times 10^{4} \pm 0{\cdot}56$		$1{\cdot}3 \times 10^{9} \pm 0{\cdot}99$	
9¶	*Lb. sakei* LMG 2313	MRSs	$2{\cdot}1 \times 10^{4} \pm 0{\cdot}64$	6·10 ± 0·03	$2{\cdot}5 \times 10^{4} \pm 1{\cdot}06$	6·08 ± 0·05	$3{\cdot}2 \times 10^{6} \pm 1{\cdot}38$§	6·00 ± 0·05
10**	*Lb. plantarum* 20	MRSs	$3{\cdot}1 \times 10^{4} \pm 0{\cdot}56$	6·09 ± 0·04	$3{\cdot}0 \times 10^{4} \pm 0{\cdot}99$	6·07 ± 0·04	$5{\cdot}0 \times 10^{6} \pm 0{\cdot}92$§	5·95 ± 0·05

*See Materials and methods.

†Reference dough: produced without adding starter LAB, incubated at 30°C.

‡Total meshophilic LAB.

§Not distinguishable from contaminating LAB.

¶Dough added of BLIS-containing cell-free, neutralized and catalase-treated supernatant from *Lb. pentosus* 2MF8.

**Dough added of BLIS-containing cell-free, neutralized and catalase-treated supernatant from *Lc. lactis* M30.

Results indicate mean ± S.D. of two independent experiments.

and/or stability of a nonsensitive strain growing in complex populations, and thus speculate on the importance of bacteriocins on regulating the interactions among microorganisms, sourdoughs produced with a combination of three LAB (BLIS or bacteriocin producer, nonsensitive and sensitive strain) were produced. Because of the higher inhibitory activity produced by this strain, only *Lc. lactis* M30 was used in this experiment. When used as a cell mixture, *Lb. plantarum* 20 and *Lb. sanfranciscensis* CB1 grew, after 21 h of fermentation, to a final cell density of $9{\cdot}4 \times 10^{8}$ and $3{\cdot}2 \times 10^{9}$ CFU g^{-1}, respectively (dough no. 6) similar to those reached at the end of individual fermentations (doughs D and F, respectively). Nevertheless we observed (data not shown) that after repeated inoculation of a sourdough started with the typical association *Lb. sanfranciscensis–Lb. plantarum*, or after the continuous use of a sourdough started with *Lb. sanfranciscensis* alone, LAB showing fast growth kinetics can prevail over *Lb. sanfranciscensis*, greatly reducing the starter performance. When *Lc. lactis* M30 was used as a starter besides *Lb. plantarum* 20 and *Lb. sanfranciscensis* CB1 (dough no. 7, Table 4), only *Lb. plantarum* 20 was inhibited, confirming the results obtained when *Lc. lactis* M30 grew in presence of only *Lb. plantarum* 20 (dough no. 3). In presence of *Lc. lactis* Q13 (not a bacteriocin producer) instead of *Lc. lactis* M30, no inhibition against *Lb. plantarum* 20 was observed (dough no. 8). In general, the sourdough fermented by the association between *Lc. lactis* Q13 and sourdough lactobacilli (dough nos. 5 and 8) or *Lc. lactis* M30 and nonsensitive lactobacilli (dough no. 7), showed, after 21 h of fermentation, higher

pH values with respect to the individual fermentations obtained by *Lb. plantarum* 20 or *Lb. sanfranciscensis* CB1 (Table 4), probably indicating a competition for carbohydrates between micro-organisms (Gobbetti *et al.* 1994). However, when *Lc. lactis* M30 was associated with a sensitive strain (dough nos. 2 and 3), similar pH were obtained with respect to the *Lc. lactis* M30 mono-culture.

The efficacy of the antimicrobial compounds produced *in situ* by *Lc. lactis* M30 and *Lb. pentosus* 2MF8 was also confirmed by extracting the inhibitory activities from doughs. Such bacteriocin preparations inhibited the indicator strains *Lb. plantarum* 20 and *Lb. sakei* LMG 2313 showing an activity of 80 and 32 AU ml^{-1}, respectively. The massive loss of activity after extraction was probably because of the binding of bacteriocins to dough components (Gänzle and Vogel 2003). No inhibitory activity was extracted from the reference dough (R) fermented for 21 h without addition of starter, indicating that the contaminating microflora growing spontaneously in the dough did not produce any antimicrobial compound.

DISCUSSION

Of 437 LAB isolated from 70 sourdoughs, five strains (two *Lb. plantarum*, two *Lb. pentosus*, and one unclassified *Lactobacillus* spp.), representing the 1·1% of the strain tested, produced antibacterial compounds, which were inactivated by proteolytic enzymes, indicating them to be bacteriocins. According to Tagg *et al.* (1976) and Jack *et al.* (1995) the five antimicrobial compounds found in this study can be regarded as BLIS as they possess bacteriocin requisites but have not yet been characterized for amino acid and encoding nucleotide sequences. In particular, due to the sensitivity to α-amylase and lipase treatments, BLIS from the two strains of *Lb. pentosus* could preliminarily be regarded as compounds belonging to the fourth class of bacteriocins, as proposed by Klaenhammer (1993). Nevertheless, as affirmed by Nes *et al.* (1996), bacteriocins of the fourth class have not yet been characterized adequately at the biochemical level and the experimental data suggest that such complex activities may be artefacts caused by interactions between constituents from the cells or the growth medium and the undefined bacteriocin activities are likely to be regular peptide bacteriocins. Indeed, proper purification of such activities leads to the isolation of regular peptide bacteriocins (Jiménez-Diaz *et al.* 1995). For the first time, BLIS production by *Lb. pentosus* isolated from sourdough is reported. Phenotypically, this species is closely related to *Lb. plantarum*, a lactic acid bacterium with potential application as a starter for the fermentation of vegetable, meat, milk, and fish products (Suma *et al.* 1998; Corsetti and Gobbetti 2002). As stated by Messens and De Vuyst (2002), and confirmed by our results, the frequency of isolating bacteriocin-producing sourdough LAB is rather low. The same finding can be extended to LAB isolated from other foods. Ennahar *et al.* (1996) showed antagonistic effects caused by antimicrobial substances other than organic acids just from six isolates of 1962 bacteria from Munster cheese. In contrast, 24% of the LAB isolates from raw milk showed inhibitory activity after neutralization and treatment with catalase (Rodríguez *et al.* 2000). From an ecological point of view, a high frequency of bacteriocin-producers may be a reflection of strong competition among micro-organisms in the ecosystem they live (Navarro *et al.* 2000). Nevertheless, during sourdough fermentation, other factors such as metabolic adaptation to the main carbohydrates available in the dough, in particular utilization of maltose resulting in a high energy yield, growth requirements which match the environmental conditions of the sourdough fermentation (Gobbetti and Corsetti 1997; Gänzle and Vogel 2003) are involved in strain adaptation besides bacteriocin production. Based on their host range inhibition, their sensitivity to enzymes and heat treatment, and the stability under different conditions, the five BLIS are considered to be different from each other. Nevertheless, all BLIS compounds were characterized by a limited inhibitory spectrum and showed no inhibition against *Bacillus* ssp., which are known to cause rope formation in bread (Röcken and Spicher 1993; Rosenquist and Hansen 1998), *Listeria innocua* and yeasts (De Vuyst and Vandamme 1994b). This is not surprising, because bacteriocins of LAB are usually inhibitory to closely related bacteria or to those competing for the same ecological niche (Klaenhammer 1988).

If the antimicrobial substances produced by sourdough LAB are resistant to baking and are active at the physical conditions of bread, they may control growth of spoilage organisms by microbial interaction (Rosenkvist and Hansen 1995). As demonstrated by the *in vitro* characterization, some BLIS compounds (e.g. those from *Lactobacillus* spp. CS1 and *Lb. pentosus* 8CF and 2MF8) show potentially interesting properties for bread-making, such as resistance to heat, stability at refrigerated storage and low pH, and activity in the presence of a high ethanol concentration. In bakery practice the sourdough is stored at +4°C for variable periods of time between repeated inoculation, while during bread baking the temperature of the crumb reaches 98°C for a period of up to 1 h (Gänzle and Vogel 2003). Moreover, in the case of sourdough bread, the pH can reach values below 4·0 at the end of fermentation and a final concentration of 10 g kg^{-1} ethanol can be reached in the dough, due to yeast fermentation (Corsetti *et al.* 1994; Gobbetti *et al.* 1995). Resistance to heat and acidity have been reported also for bavaricin A (Larsen *et al.* 1993) and plantaricin ST31 (Todorov *et al.* 1999) produced by the sourdough-isolated *Lb. sakei* MI401 and *Lb. plantarum* ST31, respectively.

The maximum activity for the five BLIS, as evaluated by the critical dilution method against *Lb. sakei* LMG2313, was obtained after 22 h of fermetation at 30°C in mMRS medium and varied between 40 and 320 AU ml^{-1}. The above combination time/temperature characterizes the type II sourdoughs employing a one-stage fermentation process (Messens and De Vuyst 2002; Messens *et al.* 2002). Thus, the five BLIS-producing LAB can be considered good candidates as starter cultures for such type of sourdough fermentation. All five BLIS showed a bacteriostatic mode of action, a characteristic which has been reported also for Plantaricin C19 (Atrih *et al.* 2001), lactocin 27 (Upreti and Hinsdill 1975) and leuconocin S (Lewus *et al.* 1992). Nevertheless, as observed by Leroy and De Vuyst (2000), the concentration and purity of the preparation, the type of broth used, the sensitivity of the indicator strain, and the density of the cell suspension applied are all factors that can influence the antibacterial mode of action of the bacteriocins. Most research on bacteriocins aimed to demonstrate the possibility to control spoilage and/or pathogenic growth in foods. The most important cause of bread spoilage is inflicted by growth of *Bacillus* species which cause ropiness. However, rope formation can occur in unacidified wheat bread (Messens and De Vuyst 2002), while sourdough fermentation can delay growth of rope-causing *Bacillus* spp. in bread (Gänzle 1998) or control growth of food-borne pathogens such as *B. subtilis* (Rosenkvist and Hansen 1995), mainly due to acetic acid production by heterofermentative LAB. Moreover, bacteriocin production by LAB may be considered as a desirable property when selecting more competitive starters for implantation and stability during repeated use (Todorov *et al.* 1999). As stated by Gänzle and Vogel (2003) 'a reproducible and controlled composition and activity of the sourdough microflora is paramount to achieve a constant quality of sourdough bread.' The same authors attributed the stable persistence of *Lb. reuteri* in an industrial sourdough fermentation to reutericyclin production (Gänzle and Vogel 2003). Among sourdough LAB, *Lb. sanfranciscensis* can be considered a key bacterium as it possesses very interesting technological properties (Gobbetti and Corsetti 1997; Gänzle and Vogel 2003). However, during repeated inoculation for the continuous use of a sourdough, *Lb. sanfranciscensis* as well as other strains eventually added as a starter, can be superseded by more competitive LAB derived from either the raw material or the bakery environment.

Based on the above considerations, with the aim to improve the implantation of technologically useful strains in the dough, we evaluated the possibility to use a bacteriocin-producer LAB in co-culture with *Lb. sanfranciscensis* CB1, a strain with excellent technological performance (Gobbetti *et al.* 1994; Gobbetti and Corsetti 1997). Besides *Lb. pentosus* 2MF8, showing the widest inhibitory spectrum among the BLIS producers characterized in this study, two *Lc. lactis* isolated from raw unmalted sorghum and barley, and previously recognized as potent bacteriocin producers (Hartnett *et al.* 2002) were evaluated. Together with *Lb. panis*, *Lb. pontis*, *Lb. reuteri*, *Lb. johnsonii*, *Lb. fermentum*, *Lb. delbrueckii*, *Lb. acidophilus*, *Lb. brevis*, *Lb. amylovorus*, and *Lb. sanfranciscensis*, *Lc. lactis* represents the dominant strains in the type II sourdoughs (Messens and De Vuyst 2002). Higher inhibitory activity, wider spectrum of inhibition and lower acidification during sourdough fermentation characterized the *Lc. lactis* strains with respect to the LAB isolated from sourdough. While acid production is an important technological feature for a starter culture and antimicrobial activity being only of secondary concern, the contrary is true for a bacteriocinogenic strain to be used as a protective culture (Messens and De Vuyst 2002) or as a co-culture (Foulquié Moreno *et al.* 2003). Bacteriocinogenic cultures could be used in association with a selected starter, provided they are able to produce their antimicrobial activity *in situ* and the bacteriocinogenic compound is active in the food matrix. Up to date no studies have been carried out on the evaluation of *in situ* bacteriocin production by sourdough LAB. Only Gänzle and Vogel (2003) demonstrated the production of the antibiotic reutericyclin during sourdough fermentation. *In situ* production of bacteriocin is influenced by growth conditions and by the metabolic state of the bacteriocin-producer (Eckner 1992). Moreover different factors, such as binding of the bacteriocins to food components, inactivation by proteases, changes in solubility and charge of the bacteriocins, as well as changes in the cell envelop of the target micro-organisms, can affect the activity of bacteriocins in foods (Gänzle *et al.* 1999). In this paper, we provide preliminary data on the *in situ* bacteriocin production and activity by *Lb. pentosus* 2MF8 and, especially, *Lc. lactis* M30. We demonstrate that the latter bacteriocinogenic strains can support the implantation of selected starter by inhibiting some of the LAB (e.g. *Lb. plantarum*) frequently prevailing during sourdough propagation. Moreover, for the first time the BLIS production by *Lb. pentosus* isolated from sourdough has been reported. Further studies are warranted to obtain direct evidence for bacteriocin production in sourdough and to evaluate the contribution the bacteriocin-producing strains can give, as 'supporting cultures' to the stable persistence of *Lb. sanfranciscensis* during the repeated use of the sourdough. Moreover, with the same approach, other technological useful co-cultures could be selected and tested to produce sourdough with a stable and persistent microflora.

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